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TISSUE FRACTIONATION AND CATECHOLAMINES

I—LATENCY AND ACTIVATION PROPERTIES OF DOPAMINE-β-HYDROXYLASE IN ADRENAL MEDULLA

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Abstract—Dopamine-β-hydroxylase was studied in fresh preparations of a granule fraction obtained from ox adrenal medulla. Preliminary observations showed that dopamine-β-hydroxylase activity could be increased up to fifteen times by means of various activating procedures, thus suggesting the existence of this enzyme in a latent form.

The latency of dopamine-β-hydroxylase was investigated by measuring the difference between the free and total activity of the enzyme. Detailed studies were made concerning the influence of the following various activating procedures: exposure to Triton X-100 and to osmotic shock, treatment with the Waring Blendor and freezing and thawing. Each of these procedures, applied in a graded manner, progressively abolished the latency of dopamine-β-hydroxylase and brought about a concomitant increase in its free activity until ultimately the total activity was attained.

The soluble activity, measured after high speed centrifugation, parallels the free activity, suggesting a progressive release of the enzyme from the granules into the soluble phase. On the other hand, dopamine-β-hydroxylase was found not to be activated by incubating or preincubating the granule fraction, whereas the catecholamines in the granules were released to a large extent during these procedures.

The results suggest that dopamine-β-hydroxylase is contained in a sac-like structure, which behaves as an osmotic system and is surrounded by a semi-permeable membrane. This could play a role as a rate limiting factor in the biosynthesis of catecholamines.

IT IS known that noradrenaline is formed from tyrosine by a series of enzymatic reactions proposed by Blaschko.[1] The final step of this biosynthesis involves the β-hydroxylation of dopamine, catalyzed by the enzyme dopamine-β-hydroxylase. This reaction was demonstrated for the first time by the use of adrenal slices and homogenates,[2, 3] following which the enzyme was partially purified by Levin *et al.*[4] Since then, the enzyme has been shown to be a copper-containing protein which requires ascorbic acid, as cofactor. In addition to dopamine, the enzyme catalyzes the side chain hydroxylation of many phenylethylamine derivatives, such as tyramine (reviewed by Kaufman and Friedman).[5] The enzyme is associated with the catecholamine-containing granules in adrenal medulla and probably also in splenic nerves and rat heart.[6–8]

The association of an enzyme with a subcellular particle can be deduced in part from investigations bearing on the latency of this enzyme; in fact, in rat liver the demonstration of latency led to the recognition of a special class of particles which contain acid hydrolases, the lysosomes.[9] In the case of lysosomes the phenomenon of

latency has been attributed to a surrounding membrane which is impermeable to both internal enzymes and external substrates. The structure-linked latency of an enzyme can be estimated biochemically as the difference between the activity of this enzyme assayed under conditions which tend to respect the integrity of the particles (free activity) and the activity determined in the presence of agents which disrupt the particles (total activity).

This report describes details of experiments dealing with the latency of dopamine-β-hydroxylase in adrenal medulla.

MATERIALS AND METHODS

Tissue fractionation

Adrenal glands of oxen were obtained from a slaughterhouse a few minutes after death and immediately chilled in ice cold 0·25 M sucrose. After dissection from the total gland, the medulla was minced and homogenized with a Potter–Elvehjem (Teflon-pestle) in 10 vol. 0·25 M sucrose. This homogenate was centrifuged at low speed (1·000 *g* for 10 min) in a Servall refrigerated centrifuge in order to eliminate cell nuclei, unbroken cells and other debris. The large granules (mitochondrial fraction) were obtained by centrifuging the first supernatant for 10 min at 27,000 *g*, and were once washed. Following a second centrifugation in the same conditions, the granule fraction corresponding to 1 g of original tissue was suspended in 20 ml of 0·25 M sucrose.

Assay of enzyme activity

Dopamine-β-hydroxylase activity was estimated by two different methods. The first is based upon a modification of the method described by Creveling *et al.*[10] using tyramine as substrate. The incubation mixture contains the following components in μmole: potassium phosphate buffer pH 5·5, 100; ascorbic acid 10; fumaric acid 12; tyramine hydrochloride 10; ATP 10; tranylcypromine sulfate 1; and 100 units catalase. To this mixture was added 0·1 ml of enzyme preparation and the final volume was adjusted to 1·0 ml with 0·25 M sucrose. The enzymic tests were performed at 25° for 15 min under these conditions, optimal activity of the enzyme was found to be linear and proportional to the amount of enzyme. The reaction was stopped by the addition of 0·1 ml of 3 M trichloroacetic acid, and after centrifugation at low speed, 0·8 ml of supernatant was loaded upon an Amberlite IRC-120 H^+ column (2·5 × 0·4 cm). After washing with 2 × 5 ml distilled water, the amines were eluted with 4 ml of 3 N NH_4OH. Octopamine (nor-synephrine) was then converted to *p*-hydroxybenzaldehyde by adding 0·6 ml 2% $NaIO_4$ to 2 ml of the eluate. After 4 min, the excess of periodate was reduced with 0·6 ml of 10% $Na_2S_2O^5$. For comparison a blank was obtained from another 2 ml aliquot by adding first, metabisulfite, and after 4 min, periodate. Absorbance was measured at 330 mμ. Total activities of dopamine-β-hydroxylase were determined in presence of 0·2% Triton X-100 following the procedure described for the lysosomes.[11] On the other hand, free activity was assayed without detergent, which is replaced by the same volume of 0·25 M sucrose. In some experiments, soluble enzyme activity was measured in the supernatant after having centrifuged the preparation at 37,000 *g* for 10 min.

In the second method for the determination of dopamine-β-hydroxylase activity, 0·5 μc of dopamine-^{14}C (sp. act. 7·07 mC/mM) was used as substrate instead of

tyramine. The incubation conditions were the same as for tyramine. The reaction was stopped by 2 ml of 10% trichloroacetic acid. After centrifugation, 10 ml of 0·2 M sodium acetate and 0·5 ml of 0·2 M EDTA were added and then brought to pH 8·4 with 3 N NH_4OH. This solution was then loaded upon a column of Al_2O_3. After washing with 30 ml distilled water, the catecholamines were eluted by 3 ml of 0·3 N acetic acid. An aliquot was chromatographed in *n*-butanol—3 N HCl on Whatman no. 1 paper (ascending). After an overnight run, the paper was cut in 1 cm sections and counted in 10 ml of Bray's solution with a Packard Tricarb liquid scintillation spectrometer.

Determination of catecholamines

The catecholamines were first extracted in acid medium. To 0·1 ml of the incubation mixture diluted with 0·8 ml distilled water was added 0·1 ml of 4 N $HClO_4$. After 30 min at 0°, the volume was brought to 3 ml with distilled water. After centrifugation which eliminated proteins, the supernatant was diluted and the catecholamines were measured fluorimetrically by the trihydroxy-indol method described by Anton and Sayre.[12]

Materials

Among the substrates or cofactors used for the enzyme assays, tyramine HCl and ATP (Adenosine-5-Triphosphate disodium salt from equine muscle) were obtained from Sigma Chemical Corp.

Labelled dopamine (3,4-dihydroxyphenylethylamine-1-^{14}C hydrobromide) was purchased from the New-England Corp. Boston, Mass.; Triton X-100 was kindly supplied by the Rohm and Haas Co. Philadelphia, tranylcypromine sulfate (Parnate) was donated by Smith, Kline and French Lab. Philadelphia.

RESULTS

In some preliminary experiments, shown in Table 1, we found that dopamine-β-hydroxylase activity was greatly increased when a granule fraction, prepared from fresh bovine medulla was incubated with a detergent such as Triton X-100. Under these conditions, the amount of octopamine formed was about fifteen times greater than without detergent. In following up this observation, it was established that various activation procedures, such as a hypotonic medium (0·025 M sucrose), 5 cycles of freezing and thawing, and homogenization for 5 min in a Waring–Blendor, were also able to enhance to a similar degree the dopamine-β-hydroxylase activity as reported in Table 1. In order to eliminate some ambiguous response related to the

TABLE 1. EFFECT OF VARIOUS PROCEDURES ON DOPAMINE-β HYDROXYLASE ACTIVITY FROM A GRANULE FRACTION

	Dopamine-β-hydroxylase activity
Activating procedures	Octopamine μmole/g/hr
None	5·3
Waring–Blendor (15 min)	70·7
Hypotonic medium (0·025 M sucrose)	85·4
Freezing and thawing (5 cycles)	69·6
Triton X-100 (0·2%)	84·4

special structure of tyramine, similar experiments were performed using dopamine-^{14}C as substrate in the incubation mixture. As shown in Fig. 1, after separation by chromatography, noradrenaline-^{14}C was formed in much larger amounts in incubation mixtures containing Triton X-100 than in those without this detergent. This first series of experiments shows clearly that dopamine-β-hydroxylase, known to be particle bound,

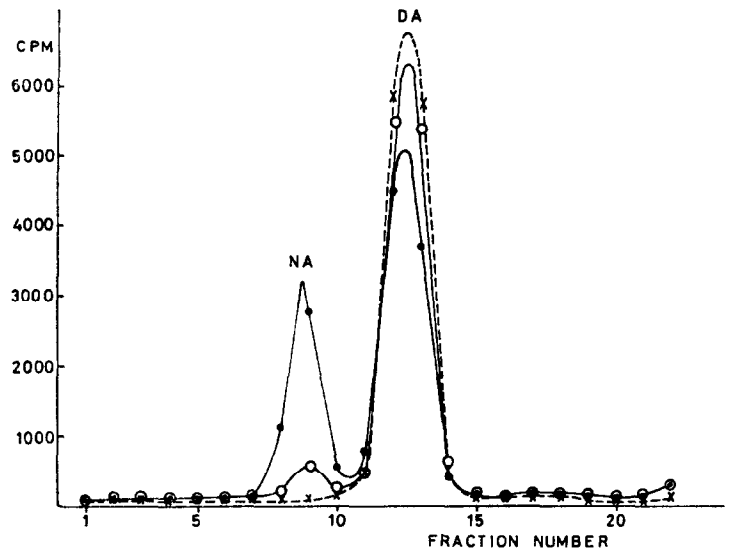

FIG. 1. Separation of noradrenaline-^{14}C and dopamine-^{14}C by paper chromatography, using butanol-HCl as solvent, from 3 different incubation mixtures containing dopamine-^{14}C and freshly prepared granule fraction treated with Triton X-100 (●), without detergent (○) and blank (×).

is contained in the granules in a latent form and can be activated by various procedures which disrupt particle membranes. Therefore, differential measurement of dopamine-β-hydroxylase can be expressed either as free activity or as total activity, the latter by adding Triton X-100 to the incubation mixture. Moreover, tyramine can be considered as an appropriate substrate for the study of some properties of the latency of dopamine β-hydroxylase.

Effect of Triton X-100

The influence of this detergent was investigated simultaneously in two different ways. First free activities of dopamine-β-hydroxylase were measured in a granule fraction by exposing it to increasing concentrations of Triton X-100. A second sample of the same preparation was centrifuged after treatment with the detergent, and the soluble activity of dopamine-β-hydroxylase and the catecholamines were determined in the supernatant. Figure 2 shows that with a Triton X-100 concentration of 0·04 per cent, the free activity of dopamine-β-hydroxylase attained the total activity. Over the range of Triton X-100 concentrations studied, the curves for the soluble activity and the catecholamines were similar and they paralleled the curve for the free activity, but were displaced to the right.

In control experiments, dopamine-β-hydroxylase from a purified soluble preparation was unaffected by various concentrations of detergent. These experiments show

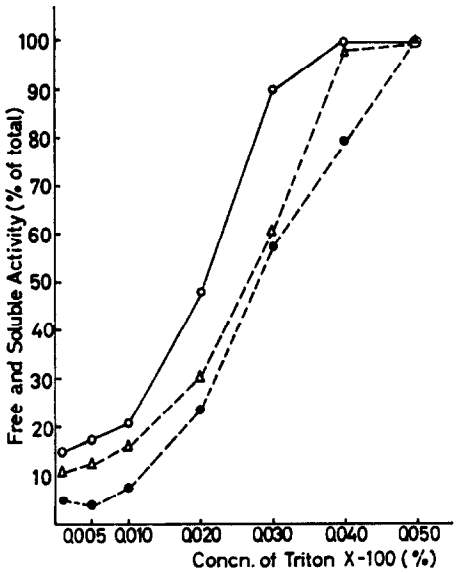

FIG. 2. Influence of various Triton X-100 concentrations on free (○) and soluble activities (●) of dopamine-β-hydroxylase and on the release of catecholamines (Δ) from a granule fraction. Results are expressed as percentages of the highest observed activities.

that 0·04% Triton X-100 represses dopamine-β-hydroxylase latency and brings about a release of this enzyme into the soluble compartment.

Effect of freezing and thawing

When a granule fraction was frozen at −10° and thawed at +4°, the free activity of dopamine-β-hydroxylase increased rapidly with the number of freezing and thawing cycles, and the catecholamines were released in a similar way, as shown in Fig. 3. In

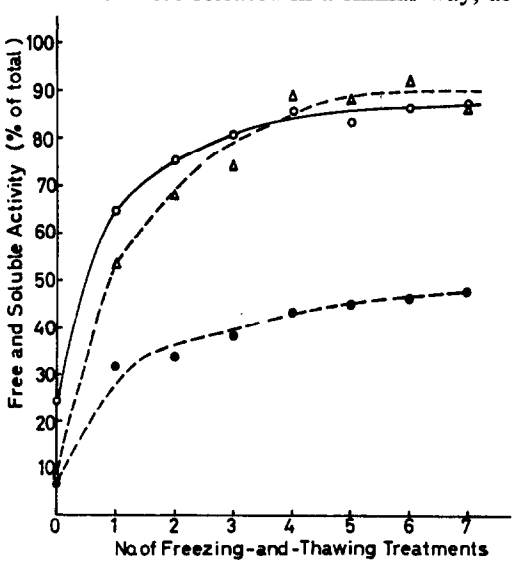

FIG. 3. Influence of freezing and thawing on free (○) and soluble activities (●) of dopamine-β-hydroxylase and on the release of catecholamines (Δ) from a granule fraction. Results are expressed as percentages of total activities.

contrast, the soluble activities increased with the number of cycles but attained only 50 per cent of the total activity after seven freezing and thawing cycles, whereas free activity reached 90 per cent of the total activity. This difference between free and soluble activities is probably due to an adsorption phenomenon of the enzyme, which will be discussed later.

Effect of treatment in the Waring–Blendor

A loss of latency was also observed when a granule fraction was treated for different times in a Waring–Blendor. This treatment produced the following results: free activity of dopamine-β-hydroxylase, 57 per cent of total activity after 1 min, 76 per cent after 2 min and 100 per cent after 10 min in the Waring–Blendor. The soluble activity reached a maximum of 70 per cent after 10 min.

Osmotic activation

To study osmotic activation, granules were exposed for 15 min at 0° to media with decreasing sucrose concentrations. After 15 min, concentrated sucrose solutions were added to bring the mixture to a final concentration of 0·25 M, then free activity, soluble activity and catecholamines were measured as described in methods. Figure 4

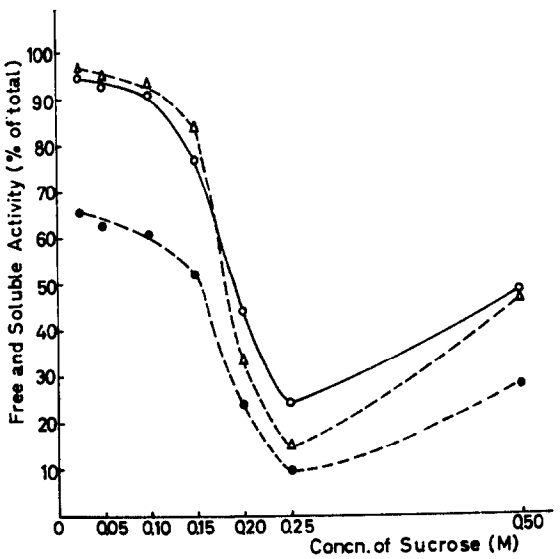

FIG. 4. Influence of decreasing osmotic pressures on free (○) and soluble activities (●) of dopamine-β-hydroxylase and on the release of catecholamines (△) from a granule fraction. Results are expressed as percentages of total activities

shows that in hypotonic media free activities increase rapidly and their osmotic activation curves are similar to those of catechoamines as found previously for freezing and thawing. The soluble activities of dopamine-β-hydroxylase paralleled the free activities but did not reach the same maximum. Slight activation was observed in hypertonic medium. Some complementary experiments have shown that increasing the sucrose concentrations to 1·5 M failed to produce activation when the granules exposed to this hypertonic medium were not brought back to the initial concentration of 0·25 M sucrose.

Effect of temperature

Two types of experiments were performed in order to show an eventual activation of dopamine-β-hydroxylase. In the first, granules were incubated at 25° with tyramine as substrate. Free and total activities were measured separately. The result was that the reaction rate for free and total activity remained linear for at least 2 hr (Fig. 5).

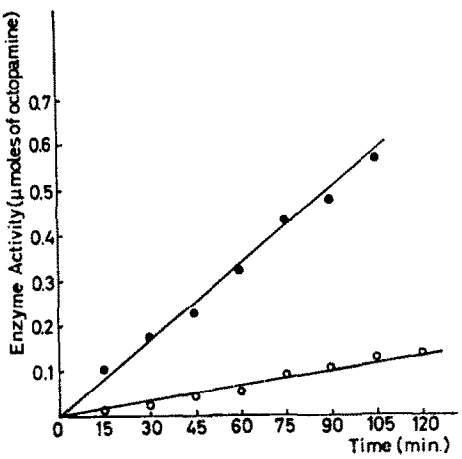

FIG. 5. Influence of time of incubation at 25° on free (○) and total activities (●) of dopamine-β-hydroxylase from a granule fraction.

Under these conditions no change of the latency was observed and the ratio of free to total activity of dopamine-β-hydroxylase was not modified. To confirm this result, a second type of experiment was performed. Granule preparations were first pre-incubated at 37° for 300 min in the medium containing catalase and buffer. From this, 3 samples were taken at different times in order to determine free and soluble activity of dopamine-β-hydroxylase and catecholamines, assays being performed as described in methods. As shown in Fig. 6, free and soluble activity of dopamine-β-hydroxylase did not increase as a function of time whereas 70 per cent of the total catecholamines was released after 300 min of preincubation. These results demonstrate that the latency of dopamine-β-hydroxylase is not affected by preincubation at 37° for periods up to 300 min whereas these same conditions render soluble a large proportion of the particle-bound catecholamines.

Effect of substrate concentration

As shown in Fig. 7, by increasing the concentration of tyramine in the incubation medium, the ratios of substrate to velocity (s/v) remain linear respectively for both the free and total activities of dopamine-β-hydroxylase. The fact that an experimental straight line was obtained for the free activities suggests that the granule is probably impermeable to the penetration of the substrate, even when concentration of tyramine was increased. Fig. 7 also shows that the K_m values are similar for the free and total activities, whereas their slopes on the base line, which represents $1/V_{max}$, are quite different. From this experiment it can be calculated that the maximum velocity (V_{max}) of dopamine-β-hydroxylase activity is about 15 times higher when Triton X-100 is added to the medium.

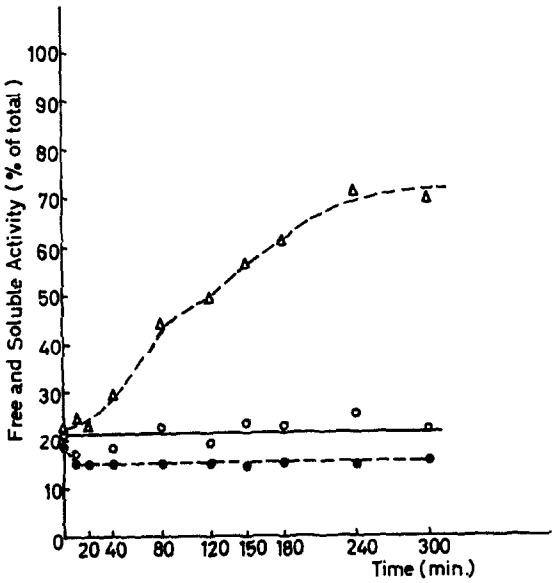

FIG. 6. Influence of pre-incubation at 37° on free (○) and soluble (●) activities of dopamine-β-hydroxylase and on the release of catecholamines (△) from a granule fraction. Results are expressed as percentages of total activities.

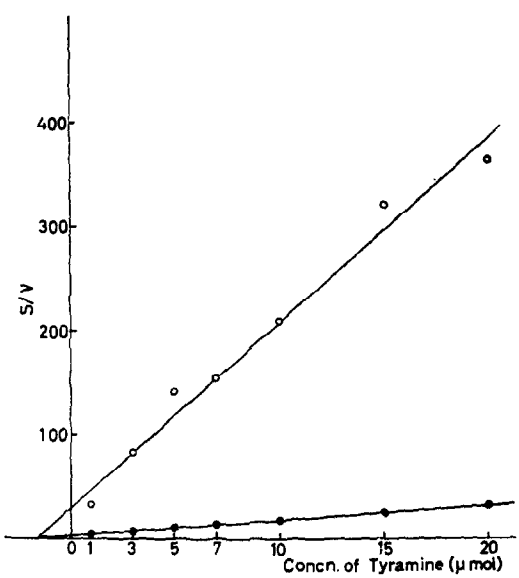

FIG. 7. Influence of substrate concentration on free (○) and total (●) activities of dopamine-β-hydroxylase. The ratio of the substrate (s) concentrations to the velocity (v) is plotted against the substrate concentration. $-K_m$ is the intercept of the experimental straight line with the base line; the slope on the line represents $1/V_{max}$.

Under our experimental conditions, a value for the K_m of dopamine-β-hydroxylase was found to be $1{\cdot}6 \times 10^{-3}$, while Creveling *et al.*[10] obtained a value of $5{\cdot}8 \times 10^{-3}$, using a purified enzyme and the same substrate.

DISCUSSION

Evidence has been accumulated that dopamine-β-hydroxylase from adrenal medulla is associated with the catecholamine containing granules.[13–16] The results described in this report show that dopamine-β-hydroxylase from bovine adrenal medulla is certainly contained in granules. This has been proved by the latency experiments from a granular fraction. Some experiments recorded elsewhere,[16] also indicate that more than 75% of dopamine-β-hydroxylase activity is found in a sedimentable form for an adrenal medulla homogenate. It is probable that all of the enzyme is particle bound in the intact cell, in which case it should be found entirely in a latent form. The small amount of unsedimentable enzyme, found in a tissue fractionation, would then reflect damage incurred by these particles during the homogenization procedure.

By using physico-chemical agents, such as freezing and thawing, homogenization in Waring–Blendor, osmotic shock or Triton X-100, the latency of dopamine-β-hydroxylase could be abolished, thereby leading to the expression of an activity some fifteen times greater than the activity normally found for untreated preparations. In increasing the concentration of substrate, the ratio of free activity to the total activity did not change. This suggests that a barrier, probably the membrane of the granules, prevents penetration or accessibility of the substrate to the enzyme. These observations, taken together, lead to the conclusion that dopamine-β-hydroxylase is contained within a sac-like structure surrounded by a membrane impermeable or semi-permeable to a high concentration of substrate. This concept, which was proposed many years ago for a special class of subcellular particles containing acid hydrolases,[17] appears to apply to lysosomes studied in many tissues in a wide variety of species.[18] However, the fact that a long time incubation or preincubation at 37° does not increase both the free and soluble activity of dopamine-β-hydroxylase, points up a difference between these particles and lysosomes, which generally lose latency during incubation at 37°. With regard to this point, some indications suggest that the internal cathepsin or some other acid hydrolases might be involved in the spontaneous disruption of the lysosomes during incubation at 37°. The fact that dopamine-β-hydroxylase-containing granules are not sensitive to thermal activation could be due to their different enzymatic composition.

The activation experiments carried out under conditions of hypotonicity point to the existence of an osmotic space in dopamine-β-hydroxylase-containing granules, which behave differently from peroxisomes.[19] Representation of these granules could be considered as an osmotic system containing various components and fluid enclosed by a semi-permeable membrane.[17]

It seems likely that the catecholamines measured in the soluble compartment provide some information about the adsorption of dopamine-β-hydroxylase from the granules. All activation experiments showed identical curves for the release of catecholamines and the free activity of dopamine-β-hydroxylase, whereas the soluble activity of the enzyme was always lower. This indicates that, if the penetration of tyramine into the granule or the release of noradrenaline and adrenaline occur in the same manner, except in the case of thermal activation, a large molecule such as

dopamine-β-hydroxylase can be only partly released from granules or, more likely, adsorbed onto other subcellular particles or granular debris. Concerning the release of catecholamines, our results are completely in agreement with the findings of Hillarp and Nilson.[20]

Our results are compatible with the consideration that the dopamine-β-hydroxylase containing particles are sac-like structure characterized by a special kind of permeability. Indeed, under the present experimental conditions of preincubation at 37°, the penetration of tyramine into the granules, as measured by the free activity of dopamine-β-hydroxylase, was probably prevented by the membrane of the granules; however the endogenous catecholamines were concomitantly released from the granules. It is quite reasonable to assume that the membrane of these granules has a different permeability for amines of different chemical structure such as dopamine, noradrenaline, adrenaline and tyramine. (Further experiments will test this hypothesis by using dopamine as the substrate.) It seems likely that the passage of amines through the membrane of the granules from the outside to the inside is equivalent to the passage from the inside to the outside, i.e. release of the catecholamines, unless one of these processes is an active one. One can propose another explanation in assuming that two compartments exist in the granules, one devoted to the β-hydroxylation and another one to the storage mechanisms.

On the basis of uptake experiments Kirshner has suggested the existence of an active phenomenon rather than an intra-granular binding mechanism, to explain the penetration of catecholamines into the granules.[13] These results were confirmed in our laboratory (unpublished data) but, even though our results are compatible with this hypothesis, it is likely that only a very small amount of catecholamines can be taken up by means of this active process, otherwise the latency of dopamine-β-hydroxylase should not occur. In this connection, it should be noted that in these uptake experiments a large amount of granules is required in order to demonstrate a significant level of labeled catecholamines in the granules.

From these results, it could be assumed that the membranes of the granules, which should prevent the penetration of tyramine and dopamine, could be considered as a rate limiting factor in the biosynthesis of catecholamines. The fact that much more dopamine than dopa is found in various tissues is compatible with this concept. Moreover, from our experiments, the maximum velocity of dopamine-β-hydroxylase was found to be much higher for a granule fraction treated with Triton X-100 than for an untreated preparation. Therefore, in the general concept of catecholamine biosynthesis, where dopamine must be transferred from the soluble compartment into the granules,[21] the presence of the dopamine-β-hydroxylase in latent form is a rate-limiting step between decarboxylation and β-hydroxylation.

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